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METHOD IN CELL SCIENCE

Production, purification and titration of a lentivirus-based vector for gene delivery purposes

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Abstract Viral vectors are valuable tools to deliver genetic materials into cells. Vectors derived from human immunodeficiency virus type 1 are being widely used for gene delivery, mainly because they are able to transduce both dividing and non-dividing cells which leads to stable and long term gene expression. In addition, these types of vectors are safe, with low toxicity, high stability and cell type specificity. Therefore, this work was aimed to produce lentivirus-based vector using a three-plasmid system. To produce this system, the eGFP marker gene was cloned into the plasmid pWPXLd. Subsequently, this vector plasmid, along with packaging plasmids, psPAX2 and envelope plasmid, pMD2.G, was co-transfected into packaging cell line (293T) using calcium phosphate method. 48 h post transfection, the constructed viral vector was harvested, purified and concentrated and stored at −80 °C for next experiments. The titration of the vector was carried out, using ELISA, flowcytometry, and fluorescent microscopy. Finally, transduction of HEK-293T, CHO, HepG2, MCF-7, MEFs and Jurkat cell lines was carried out with indicated cell numbers and multiplicities of infections of the vector in the presence of polybrene. Using this system, high titer lentivirus at titers of up to 2×10^8 transducing units/ml (TU/ml) was successfully generated and its transduction efficacy was improved by seven to over 20-fold in various cell types. We demonstrate the applicability of this vector for the efficient transduction of dividing and non-dividing cells, including HEK-293T, CHO, HepG2, MCF-7, MEFs and Jurkat cell line. Transduction efficiency yielded titers of (6.3 ± 1.2) 10^5 TU/ml. Furthermore, lentivirus transferred transgene was expressed at high level in the target cells and expression was followed until 90 days after transduction. Thus, the vector generated in this work, might be able to deliver the transgene into a wide range of mammalian cells.

Keywords Lentivirus-based vector · Transfection · Transduction

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Introduction

Numerous researches have been carried out to establish the genes delivery methods to introduce genes of

interest (GOI) into the mammalian cells for stable and efficient expression of the GOI. These valuable researches, result in the expression of a wide range of methods such as Ca-phosphate precipitation, DEAE-dextran transfection, electroporation, and liposome for delivery of DNA into mammalian cells. None of these transfection methods are comparable with viral methods with respect to simplicity, efficiency, and straightforwardness.

Among the virus based methods, the lentivirus based vectors are very efficient gene delivery vehicles and are able to provide long-term gene expression. Along with the increasing use of lentiviral vectors, the production and purification procedures for the generation of these highly efficient vectors need to be developed (Segura et al. 2010). It has been shown that a variety of production systems efficiently generate transduction competent lentiviral particles, including transient transfection of mammalian cells (Naldini et al. 1996) and use of stable packaging cell lines (Kafri et al. 1999). Transient transfection approach that uses multiplasmids continues to be the most common techniques for lentiviral production because it is faster, simpler and more versatile than the other methods. Developing of stable packaging cell line for lentiviral production is a tedious and time consuming process and also, cytotoxicity of viral components or transgenes can be a serious barrier (Bartz et al. 1996; Konvalinka et al. 1995; Li et al. 1995; Miyazaki et al. 1995). Furthermore, transient-transfection systems permit testing various transgene of interest and envelope proteins with alternative cell tropism in a relatively short time (Sena-Esteves et al. 2004). Many detailed protocols are available describing production of lentiviral vectors by multiplasmids transient transfection of human embryonic kidney (HEK) 293 T cells using different transfection reagents such as calcium phosphate or lipofectamine (Follenzi and Naldini 2002; Tiscornia et al. 2006; Salmon and Trono 2001). In these protocols producer cells are grown as monolayer in 10 or 15 cm culture dishes in the presence of 10 % fetal bovine serum (FBS). Following the successful transfection, 10–15 ml of supernatant containing 10^5–10^7 infective lentiviral particles per ml (IVP/ml) can be harvested at day 2 and 3 post transfection. Subsequently, the harvested supernatants are pooled, filtered through 0.45 nm filter to remove cell debris. Increased titers can be achieved by ultracentrifugation or ultrafiltration of virus-containing cell culture supernatant, using a protocol initially developed for concentration of MoMLV vectors (Burns et al. 1993). Pseudotype lentiviral particles containing the Vesicular Stomatitis Virus glycoprotein (VSV-G) has shown to be stable and to withstand concentration by ultracentrifugation without significant loss in titer (Naldini et al. 1996; Bartz and Vodicka 1997). This facilitates the generation of highly concentrated vector stocks for applications that need high titer viral stock like in vivo experiments. Concentration protocols based on ultrafiltration were also established for HIV-1 based vectors. Reiser et al. (1996) have developed a facile ultrafiltration procedure based on centriprep units (Millipore, Bedford, MA) to concentrate HIV-1 pseudotypes. Good recoveries have been reported based on this procedure (Reiser 2000).

Large-scale production of Lentiviral vector for both in vivo and in vitro applications is very challenging due to lack of simple and cost-benefit procedures that could be capable of efficient processing of large volumes of cell culture supernatant in a reasonable amount of time.

The traditional ultracentrifugation approaches are limited in terms of their capacity to handle large volumes and costs. Overnight low speed centrifugation has been used to process small to large volumes of up to l000 ml of virus containing supernatant produced in Nunc Cell Factories (Nalge Nunc, Naperville, IL) (VandenDriessche et al. 2002). However this low-speed approach is time consuming and has low efficiency.

There is an emerging need for quick, reproducible, efficient, cost-benefit and less laborious procedures that could rapidly reduce the volume of the supernatant need to be processed. Pham et al. (2001) have developed a simple and gentle precipitation method that could rapidly reduce the volume of both MoMLV and lentiviral vectors involving the precipitation of pH-adjusted viral supernatants with calcium phosphate, low-speed centrifugation and dialysis. The volume can be decreased from 300 to 10 ml with 50–100 % recovery rate. Zhang et al. (2001) have presented an alternative precipitation method based on poly-L-lysine (PLL). In this method, viral vector containing supernatants were mixed with PLL, incubated at 4 °C for 30 min, and centrifuged at 10,000*g* for 2 h; reports have shown that recovery rate for processing of up to 3 liters of cell culture was 26-32 %.

The aim of this study was to produce high titer lentivirus based vectors that could be used for gene delivery purposes.

Materials and methods

Cells and culture

Chinese hamster Ovary (CHO), Human Embryonic Kidney (HEK) 293T, Human hepatocyte carcinoma (HepG2), human breast adenocarcinoma (MCF-7), Mouse embryonic Fibroblast (MEF) and Jurkat cell line were obtained from the National Cell bank of Iran (NCBI) and were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FCS (Gibco, Life Technologies, Carlsbad, CA, USA) and 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies) and 2 mM glutamine (Life Technologies). The cells were plated in 75 cm^2 flasks (Orange Scientific, Braine-l'Alleud, Belgium) and cultured in DMEM at 37 °C in a humidified atmosphere of 95 % air/5 % CO_2 with medium exchanges every 3 days. Following 2 weeks of incubation, the cells were suspended in and then harvested from the medium.

Vector generation

Lentivirus-based vector expressing GFP was generated by transient co-transfection of 293T cells with a three-plasmid combination, HBS2X. Briefly, a 100 mm dish of non-confluent 4×10^6 cells were transfected with. 3 μg of psPAX2 (Addgene, Cambridge, MA, USA, Plasmid 12260), 1.5 μg of pMD2G (Addgene, Plasmid 12259), and 6 μg of lentiviral vector plasmid (pWPXLd) mixed with 500 μl diluent (660 μL TE 0.1X, 350 ml ddH_2O, 113 μL $CaCl_2$ 2.5 M, 5 mM HEPES, 150 mM NaCl, pH = 7.05) and incubated at room temperature (RT) for 15 min. Following the incubation, 1,100 ml HBS2X was added to the mixture, incubated at RT for 5 min and added to partially confluent HEK 293T cells. 36, 48 and 72 h's post-transfection, Lentivirus containing supernatant was harvested and used for next experiments.

Concentration of lentivirus-based vector

Two concentration methods were used to concentrate vector containing supernatant. In ultracentrifuge-based method, the supernatant was spinned at 47,000*g* for 2 h and the pellet was resuspended in 100–200 μL PBS 1X. In the precipitation-based method, 8.5 % poly-ethyleneglycol (PEG) 8,000 and 0.4 M NaCl were added to lentiviral vector containing supernatant and incubated at 4 °C for 5 h along with mixing every 20 min. Subsequently, the mixture was pelleted at 7000 g for 15 min and the pellet was re-suspended in 100–200 μL of PBS 1X.

Titration of lentivirus vector

Titration of eGFP expressing lentivirus vector was carried out by transduction of HEK 293T cells. Briefly, 10^6 HEK 293T cells were plated onto 24 well plate in 1 ml medium per well. Subsequently, these cells were transduced with four and sixfold dilutions of the vector and 3 days post transduction, the eGFP expressing cells were analyzed using fluorescent activated cell sorting (FACS) method for each dilution. The titer was calculated based on following formula:

Cell number × 2 (doubling factor in 24 h) × % $eGFP^+$ cells × 1,000/μL virus.

Cell transduction

Transduction of HEK-293T, CHO, HepG2, MCF-7, Jurkat cell lines and Mouse embryonic Fibroblast (MEFs) were carried out with indicated cell numbers and multiplicities of infections (MOIs) of the vector for 2 h in the presence of 8 μg/ml of polybrene. Cells were prepared so that theywere growing exponentially and were no more than 70–80 % confluent before transduction. At day 1, 1.5 x 10^4 cells added in fresh medium to the wells of 96-well plate (Nunc, Roskilde, Denmark) followed by incubation for 18-20 hours at 37 °C in a humidified incubator in an atmosphere of 5–7 % CO_2. At day 2, medium removed from wells and to each well 110 μL medium and polybrene to a final concentration of 8 μg/ml were added. Ten μL of lentiviral vectors was added to appropriate wells, gently mixed and incubated for 18–20 hours at 37 °C in a humidified incubator in an atmosphere of 5–7 % CO_2. Due to toxicity concerns of lentiviral vectors, MEFs were incubated for 2 to 4 hours before changing the medium containing lentiviral vectors.

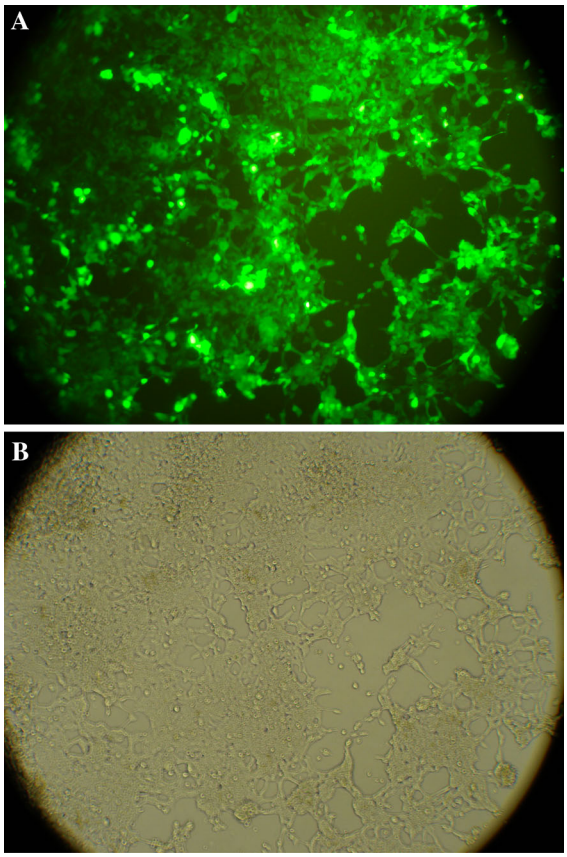

Fig. 1 GFP expression in HEK-293 T cells 3 days posttransfection of HEK-293 T-cells with the lines with the plasmids, phase contrast microscopy (**a**), fluorescent microscopy (**b**), and light microscopy; showed that due to transfection with the plasmids,GFP expressing lentiviral vector was produced in more than 70 % of the cells

In vitro lentivirus-based vector transduction efficiency

Cells were seeded in 96-well plates (Nalge Nunc) at a density of 2×10^3/well, and 0.5 ml DMEM with 1 % penicillin–streptomycin and 15 % FBS was added to each well. Viral particles at an MOI of 2.5, 5, 10, 20, 50, and 100 were added to the wells. Following incubation at 37 °C in 5 % CO_2 for 24 h, the virus-containing medium was removed and replaced with 0.5 ml fresh culture medium per well. The transduction efficiency was determined daily with an inverted fluorescent microscope (DMI3000B; Leica, Wetzlar Germany) and was quantified by measuring the GFP-expressing cells as a percentage of the total number of visible cells.

Results

Construction of GFP expressing lentiviral vector using of HEK-293 T-cells

HEK 293T cell line was transfected with the plasmids pWPXLd, pMD2G, psPAX2, to produce a GFP expressing lentiviral vector. 36 hours post-transfection, fluorescent microscopy showed that GFP was expressed in more than 70 % of these cells (Fig. 1).

Concentration and titration of GFP expressing lentiviral vector

Using both ultracentrifuge-based and precipitation-based methods, lentiviral containing supernatant was concentrated and subsequently used for titration. 3 days post transduction of HEK 293T cells with 4 and sixfold dilutions of the vector, FACS analysis showed that after only one-round of concentration by ultracentrifugation at 50,000*g*, titers of (2.2 ± 1.0) 10^8 TU/ml on 293T cells were routinely achieved.

Time kinetics of gene expression in lentivirus based vector using of HEK-293 T cells

To analyze the kinetics of lentiviral vector productions in further detail and to investigate the possibility of GFP expression over time, HEK 293T cells were continuously passaged after infection and the percentage of GFP-expressing cells was determined by FACS analysis on days 4, 6, 10, 14, 17, 24, and 90 post-infection. In both cell lines, the percentage of GFP-positive cells was stable throughout the culture period and the percentage of GFP-positive cells correlated with the promoter. These results indicate that transgene expression levels are stable for at least 90 days (Fig. 2).

Transduction of CHO, Jurkat, HepG2, MEFs and MCF-7 cell lines

Our results showed that the cell lines HEK 293T, CHO, Jurkat, HepG2, MCF-7 and MEFs were efficiently transduced with the vector. 72 hours post transduction, the transduction rate was analyzed using direct fluorescence microscopy technique. Based on the results, GFP was expressed in more than 95 % of the transduced cells including MCF-7 (Fig. 3), Jurkat

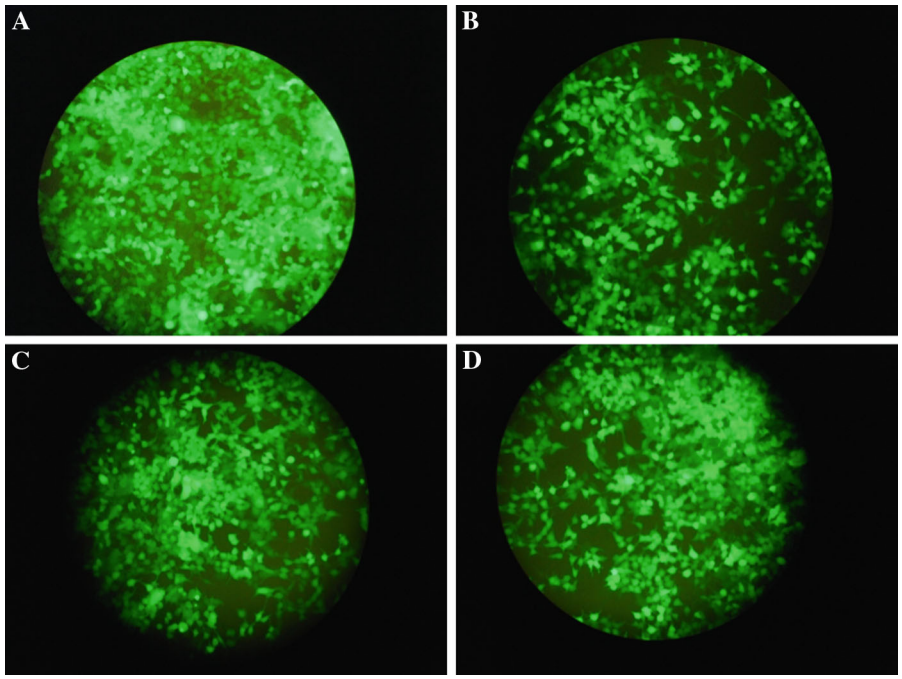

Fig. 2 Time kinetics of gene expression lentivirus vector in HEK-293 T cells. Figures **a**–**d** show efficient long term GFP expression in HEK-293 T cell lines 3–90 days post transduction. **a** day 3, **b** day 10, **c** day 45, **d** day 90

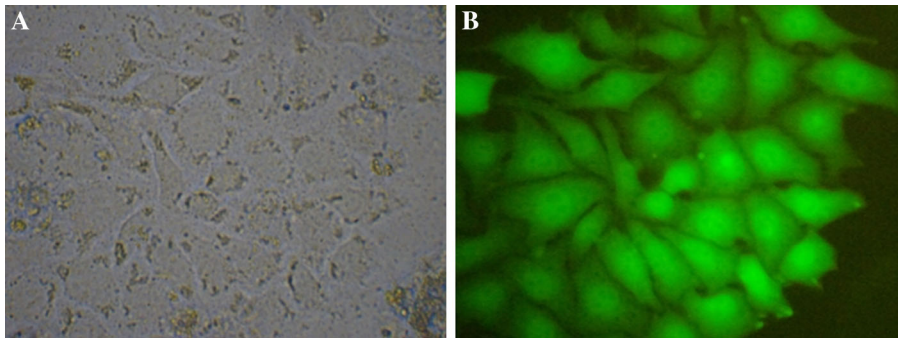

Fig. 3 Transduction of MCF-7 cells with GFP expressing lentiviral vector. MCF-7 cells were transduced with the vector. 72 h post transduction, light microscopy (**a**) and fluorescent microscopy (**b**) results showed that 95 % of the cells were transduced with the vector

(Fig. 4), HepG2 (Fig. 6), and MEFs (Fig. 7). Base on the FACS analysis, the transduction rate of CHO (Fig. 5) and HEK 293T cells was 99.2 % and 96 %, respectively.

Discussion

Lentivirus-based vectors constitute a valuable tool for gene transfer technology. The vectors are traditionally produced by transient cotransfection of human embryonic kidney 293T cells using three to four different plasmids (Addgene plasmid repository). A successful gene delivery experiment using lentivirus-based vector depends on a good co-transfection of constituting plasmids on packaging cell line and an efficient concentration method. In this work, for generation of this vector, we used a 3-plasmid system in the 293 T cell line. Based on our results 72 h post-transfection, fluorescent microscopy showed that this vector was produced in more than 70 % of these cells (Fig. 1).

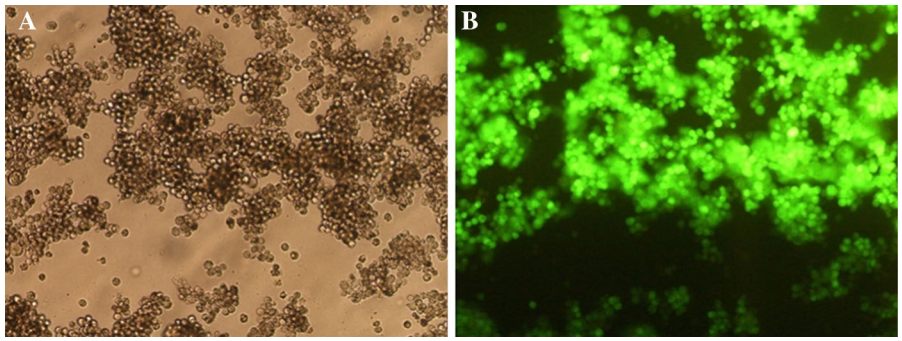

Fig. 4 Transduction of Jurkat cells with GFP expressing lentiviral vector. Jurkat cells were transduced with the vector. 72 h post transduction, light microscopy (**a**) and fluorescent microscopy (**b**) results showed that the 95 % of the cells were transduced with the vector

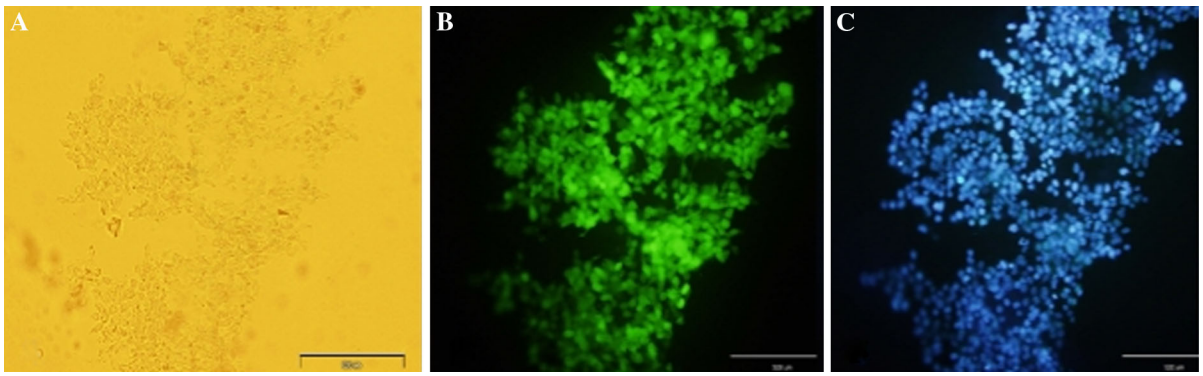

Fig. 5 Transduction of CHO cells with GFP expressing lentiviral vector. CHO cells were transduced with the vector. 72 h post transduction, light microscopy (**a**), fluorescent microscopy (**b**), and DAPI staining (**c**) results showed that 99.2 % of the cells were transduced with the vector

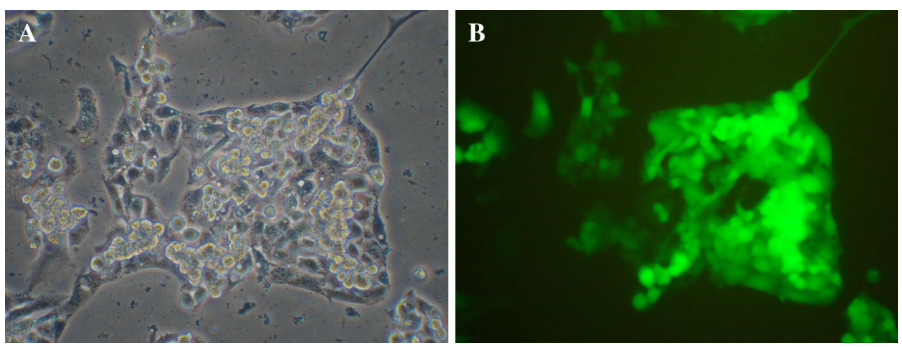

Fig. 6 Transduction of HepG2 cells with GFP expressing lentiviral vector. HepG2 cell were transduced with the vector. 72 h post transduction, light microscopy (**a**) and fluorescent microscopy (**b**) results showed that 95 % of the cells were transduced with the vector

Studies have shown that the efficiency of lentivirus-based vector can be significantly improved by concentration and removal of impurities (Zimmermann et al. 2011). Typical virus titers range from 10^6 to 10^7 transducing units per milliliter (TU/ml). Increased titers can be achieved by concentration of the culture supernatants by ultracentrifugation (Naldini et al. 1996). Pseudotyped lentiviral particles containing the VSV-G glycoprotein used in this work, have been shown to be quite stable and withstand concentration

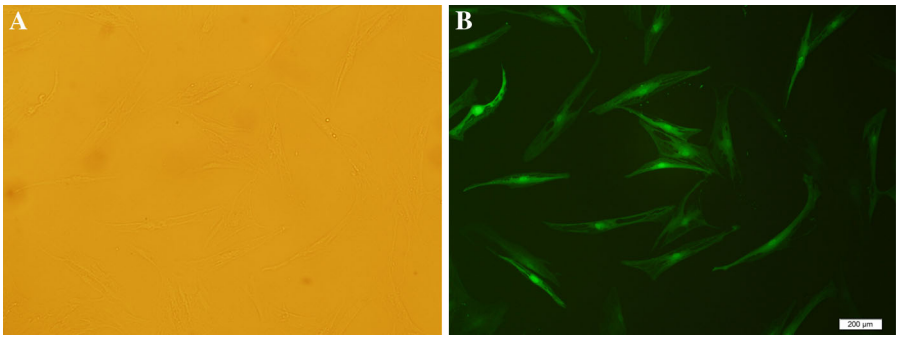

Fig. 7 Transduction of MEFs with GFP expressing lentiviral vector. Mouse embryonic fibroblast cells were transducer with the vector. 72 h post transduction, light microscopy (**a**) and fluorescent microscopy (**b**) results showed that vast majority of the cells were transduced with the vector

by ultracentrifugation without significant loss in titer (Fig. 2). This might open up a way to generate highly concentrated vector stocks for in vivo applications in our laboratory.

To analyze the kinetics of the transgene (GFP) expression, HEK 293T cells were transduced with the viral vector and expression of GFP in the expressing cells was followed up to more than 3 months. As Fig. 2 shows, frequent passage of the cells had no effect on GFP expression indicating that these cells became transgenic.

Our results showed that the vector transduced the dividing cells CHO, Jurkat, HepG2, and MCF-7 and MEFs efficiently with the rate of more than 95 % after 72 h. These cells are dividing cells and are widely used in different research fields. It has been demonstrated that GFP expressing lentivirus efficiently deliver transgene into HepG2 cells and that two lentiviral constructs are highly efficient for silencing apoB expression in these cells (Liao and Ning 2006). Accordingly, Jurkat cell is used to study the expression of various chemokine receptors susceptible to viral entry, MCF-7 is used in cancer research, and CHO is being used especially when long-term, stable gene expression and high yields of proteins are required. Therefore, the vector generated in this work would be a promising tool in the expression of therapeutic genes in both dividing and non-dividing cell lines in vitro and may be in vivo as well.

Conclusion

Our results showed that GFP expressing lentivirus-based vector, generated in this study, can transduce efficiently dividing cells CHO, Jurkat, HepG2, MCF-7 and MEFs. Conclusively, the method used in this work is a simplified, easy to set up, reproducible, efficient, cost-benefit and less laborious method for generation of a lentivirus-based vector that could be used in a wide range of gene delivery experiments.

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